Role of Cytokines and Reactive Oxygen Intermediates in the Inflammatory Response Produced by Sulfur Mustard . . . A Progress Report

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ABSTRACT

Background. Cytokines play a major role in both acute and chronic inflammatory processes, including those produced by sulfur mustard (SM). In situ hybridization of the mRNA of various cytokines with radiolabeled antisense RNA probes enables us to visualize under the microscope which cells in tissue sections of SM lesions are producing which type of cytokine. This technique, therefore, demonstrates cell function histologically, even though the cells are no longer alive at the time of analysis.

Cytokines from infiltrating phagocytes. We have successfully demonstrated the mRNAs of four major cytokines in developing and healing rabbit SM lesions: Interleukin 1 beta (IL-1 beta), neutrophil attractant /activation protein 1 (NAP-1 or IL-8), monocyte chemoattractant (activating) protein 1 (MCP-1), and GRO, which is macrophage inflammatory protein 2. The macrophage/fibroblast group in the lesions contained the mRNA of all four cytokines, and granulocytes contained the mRNA of IL-1 beta and NAP-1. More cytokine producing cells were present in the peak lesions than in healing lesions.

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Cytokines from dermal epithelium. In SM lesions (but not in normal skin), epithelial cells of the surface and/or of hair follicle epithelium contained the mRNA of NAP-1 and MCP-1. Evidently, SM induces such epithelial cells to produce these two chemotactic/activating cytokines, which, in turn, chemoattract polymorphonuclear or mononuclear phagocytes and locally activate the fibroblasts. These three cell types, then, produce more cytokines, thereby perpetuating the inflammatory process.

H_2O_2 production. We have developed a histochemical test for the production of H_2O_2 in tissue sections of SM lesions. Intact granulocytes, as well as those recently dead in vivo, were major producers of H_2O_2. Cells in the macrophage/fibroblast group also produced it in lesser amounts. No tissue destruction was seen adjacent to the cells producing H_2O_2; in fact, the regenerating epithelium grew right under crust-cells that were producing large amounts of H_2O_2.

INTRODUCTION

Cytokines are locally acting peptide growth factors that play a major role in the inflammatory process induced by sulfur mustard and other irritants (1). There are many cytokines, and there is much duplication of function among them. Also, some cytokines mainly participate in the early lesions, others in the peak lesions, and still others in the regressing lesions. Finally, each cytokine has its own specific receptors, so that up- and down-regulation of cytokine receptors on cells in inflammatory lesions are just as important as the up- and down-regulation of cytokine production.

Irritants, including sulfur mustard (SM), are thought to release primary cytokines, such as IL-1 and TNF, from epidermal cells (Figure 1). These primary cytokines, in turn, stimulate the production of other cytokines by cells in the dermis (fibroblasts, histiocytes/macrophages, endothelial cells, and mast cells). Only then is the inflammatory process fully developed.

Mast cells seem to be specialized cells that trigger the inflammatory response irrespective of the involvement of the epidermis. They are extremely sensitive to all skin irritants, including sulfur mustard, which causes mast cells to release histamine and eicosanoids for the immediate inflammatory response as well as cytokines for both immediate and delayed responses.

Thus, there is a dual mechanism responsible for the initiation of SM skin lesions: (a) primary cytokines (and probably other factors) released from the irritated or injured epidermal cells, and (b) histamine (and other products) released from irritated or injured mast cells.

MATERIALS AND METHODS

In situ hybridization methods in tissue sections are now established procedures (2,3). The most generally successful method involves the hybridization preparation of specific antisense RNA probes to hybridize with the specific cytokine mRNA present in individual cells. We use the method of Mueller (4,5). We prepare for each cytokine an antisense ^{35}S-labeled RNA probe and a sense (control) ^{35}S-labeled RNA probe. Then, after prehybridization, we hybridize at 45°C for 17 hrs (4,5).

We developed a method for identifying cells producing hydrogen peroxide in tissue sections (of SM lesions) (6). It involves the oxidation of diamino benzidine to a colored reaction product. The complete inhibition of this reaction by catalase demonstrates its specificity for H_2O_2.

RESULTS

Figure 1 illustrates an overview of our research program. From the literature (see 7), it seems that irritants, such as sulfur mustard, have direct effects on surface epidermal cells, hair follicle cells and mast cells. SM may also irritate capillary endothelial cells, local fibroblasts and local histiocytes. These stimulated cells then release stored primary cytokines, such as IL-1 alpha, and start producing other primary cytokines, such as IL-1 beta and TNF alpha. To produce them, the cells manufacture cytokine mRNAs, which are identified by in situ hybridization techniques.

The primary cytokines up-regulate adhesion molecules (the selectins and the integrins) on vascular endothelial cells and leukocytes (8). This enables the emigration of leukocytes into the area. The primary cytokines also up-regulate the production of secondary cytokines in almost every cell-type present, including the infiltrating cells, which are now plentiful. Receptors for each cytokine (and each adhesion molecule) are also up-regulated. Since many of these cytokines are chemotactic, they are a major cause of the cell infiltration in inflammatory lesions.

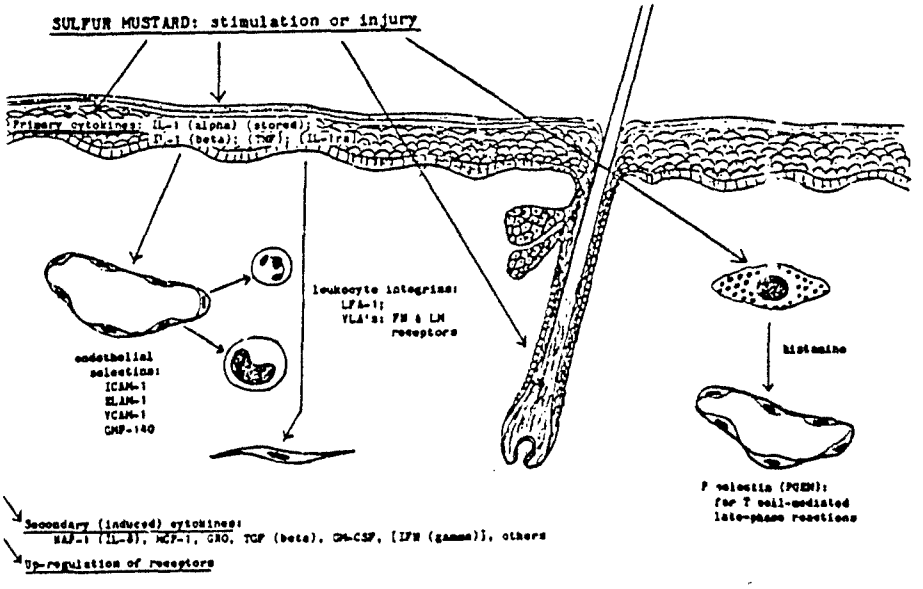

Figure 1. An overview adapted from Thomas S. Kupper (7) illustrating the local roles that cytokines play in the inflammatory process.

Figure 2 shows that, in SM lesions, the number of cells containing mRNA for IL-1 beta and NAP-1 (IL-8) peaked at two days. The number of cells containing mRNA for IL-1 beta and GRO was also high at 4 and 8 hours. The number of cells containing mRNA for MCP-1 was high at one day (and possibly earlier) and declined steadily thereafter.

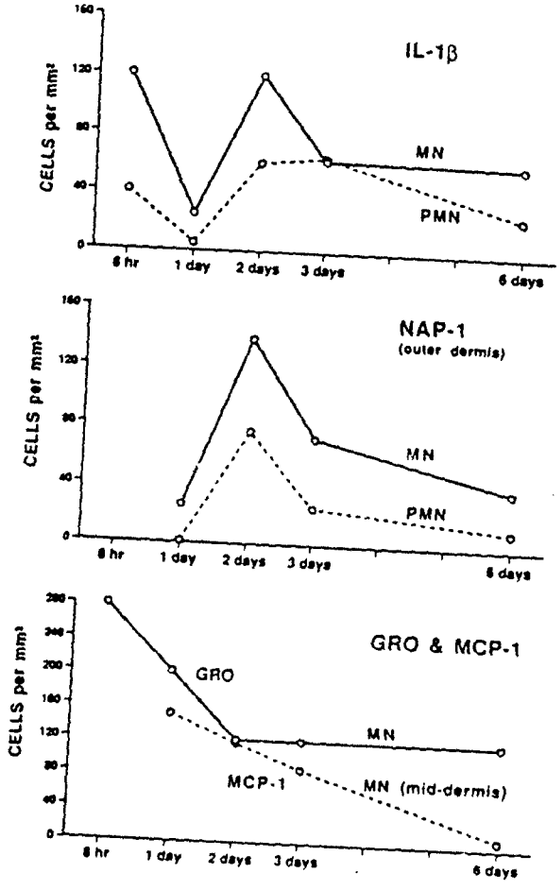

Figure 2. Cytokine mRNAs in developing and healing SM lesions, produced in the skin of rabbits. Histochemical in situ hybridization studies with antisense ^{35}S-RNA cytokine probes. MN = cells of the macrophage/fibroblast group. PMN = granulocytes.

Figure 3 illustrates MCP-1 mRNA in the macrophage/fibroblast group of cells in a 3-day SM lesion. Sense MCP-1 RNA probes labeled few if any macrophage/fibroblast cells. In normal skin, very few cells label with the antisense MCP-1 RNA probe.

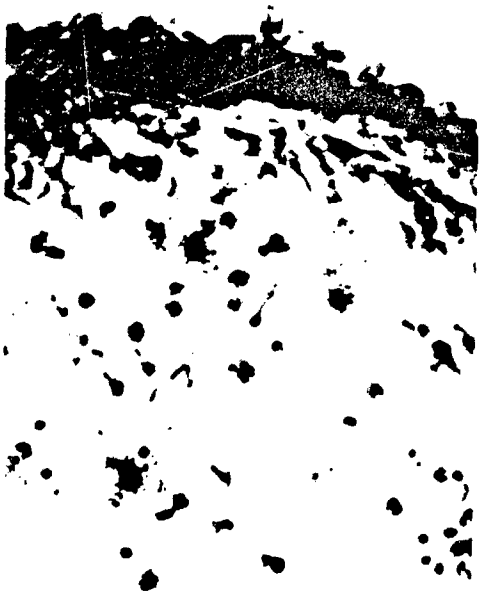

Figure 3. MCP-1 mRNA in a rabbit 3-day dermal SM lesion. This is an autoradiograph of a frozen section of a cold-formalin-fixed SM lesion, hybridized in situ with ^{35}S-labeled antisense MCP-1 RNA and counterstained with Giemsa. X 500.

Figure 4 illustrates a hair follicle cell labeled with antisense NAP-1 (IL-8) mRNA in a 3-day SM lesion. SM evidently caused the keratinocytes in this hair follicle to produce much NAP-1 mRNA. Sense NAP-1 did not label any hair-follicle cell. No hair-follicle cells in normal skin label with either the antisense or sense probe. The hair follicle in this photograph was labeled more heavily than most; in fact, many hair follicles in SM lesions were not labeled at all.

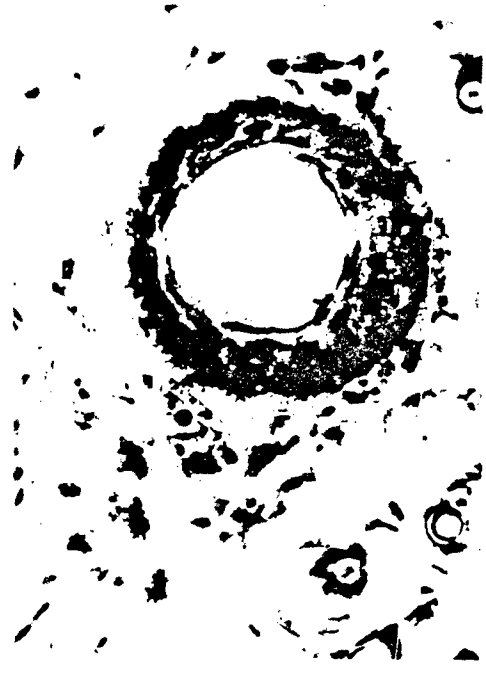

Figure 4. A hair follicle cell labeled for NAP-1 (IL-8) mRNA in a rabbit 3-day dermal SM lesion. This is an autoradiograph of a frozen section of a cold-formalin-fixed SM lesion, hybridized in situ with ^{35}S-labeled antisense NAP-1 RNA and counterstained with Giemsa. X 500.

Figure 5 shows H_2O_2 production in a tissue section of a 3-day SM lesion. Intact granulocytes in the tissues and in the crust oxidized the diaminobenzidine (DAB) substrate, producing an insoluble brown polymer. The disintegrating granulocytes in the crust also oxidized DAB. Catalase (0.15 to 1.5 mg/ml) inhibited the reaction, identifying H_2O_2 as the oxidant of the DAB. Regenerating epidermal cells (migrating from hair follicles) have grown under the H_2O_2-producing crust. These new epidermal cells were apparently totally resistant to any toxic effect that H_2O_2 might have. A manuscript describing this histochemical test for H_2O_2 has just been submitted to the Journal of Leukocyte Biology (6). Evidently, most of the H_2O_2 in the granulocytes is derived from the dismutation of superoxide, and nitric oxide does not oxidize DAB (6).

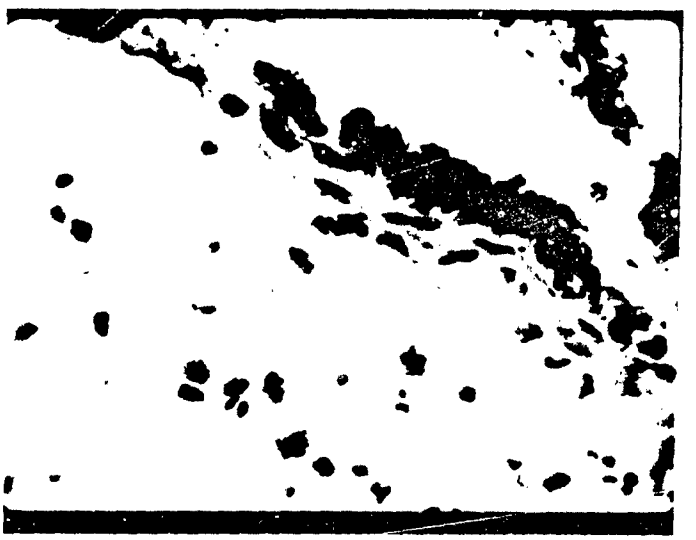

Figure 5. H_2O_2 production in a cold-formalin-fixed tissue section of a rabbit 3-day SM lesion. A brown-orange reaction product (which is difficult to identify in black and white photographs) is present in most of the live and disintegrating cells in the crust and in several granulocytes in the dermis. Diaminobenzidine (DAB) and glucose, incubated at 37°C for 5 hours at pH 6.8, and counterstained with Giemsa. X 625.

DISCUSSION

These in situ hybridization techniques for the cytokine mRNAs were performed on fixed-frozen tissue sections. We hope to confirm these results using paraffin-embedded or plastic sections for better identification of the individual cells in the SM lesions.

The immunohistochemistry of these cytokine proteins in SM lesions has, to date, not been successful in our laboratory -- probably because these proteins are in low concentration and are short-lived. On tissue sections of rabbit SM lesions, we have used mouse monoclonal antibodies to rabbit IL-1 alpha and IL-1 beta. On tissue sections of discarded human skin (from mastectomies) exposed to SM for 3 hours in vitro, we have used mouse monoclonal antibodies to human MCP-1 and NAP-1 and rabbit polyclonal antibodies to human IL-1 alpha, IL-1 beta, TNF alpha, and IL-6. Both the ABC [avidin-biotin-(peroxidase)-complex] kit (from Vector Laboratories, Burlingame, CA) and the immunogold-silver intensification kit (from Goldmark Biologicals, Phillipsburg, NJ) were employed. Both kits worked fine with our positive controls (which were surface antigens for macrophages and lymphocytes).

Keratinocytes immunostain nonspecifically. Therefore, we could not identify the IL-1 known to be stored in the epidermis. We hope, however, to show (using the ELISA technique) that more IL-1 is released into culture fluids by SM-exposed full-thickness human skin explants than by unexposed explants.

Finally, we have obtained a goat antibody to rabbit VCAM-1 from Dr. Myron I. Cybulsky, of Brigham and Women's Hospital, Boston, MA 02115, and expect to receive a goat antibody to rabbit ICAM from Dr. Barry A. Wolitzky, Hoffmann-La Roche, Nutley, NJ 07110. These antibodies will be used with the Vector and Goldmark kits to identify the up-regulation of adhesins by cells in SM lesions.

In conclusion, we have identified the mRNAs of IL-1 beta, NAP-1 (IL-8), MCP-1 and GRO in rabbit dermal SM lesions in various stages of developing and healing, substantiating some of the interactions illustrated in Figure 1. We have not yet proven that SM releases the primary cytokine, IL-1, from the epidermal cells. In the near future, we hope to show (with our new antibodies to rabbit VCAM and ICAM) that SM up-regulates these important adhesins.

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